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Membrane Insertion of the F-Pilin Subunit Is Sec Independent but Requires Leader Peptidase B and the Proton Motive Force

NADIM MAJDALANI* AND KARIN IPPEN-IHLER†

Department of Medical Microbiology and Immunology, Texas A&M University Health Science Center, College Station, Texas 77843

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F pilin is the subunit required for the assembly of conjugative pili on the cell surface of *Escherichia coli* carrying the F plasmid. Maturation of the F-pilin precursor, propilin, involves three F plasmid transfer products: TraA, the propilin precursor; TraQ, which promotes efficient propilin processing; and TraX, which is required for acetylation of the amino terminus of the 7-kDa pilin polypeptide. The mature pilin begins at amino acid 52 of the TraA propilin sequence. We performed experiments to determine the involvement of host cell factors in propilin maturation. At the nonpermissive temperature in a $LepB_{ts}$ (leader peptidase B) host, propilin processing was inhibited. Furthermore, under these conditions, only full-length precursor was observed, suggesting that LepB is responsible for the removal of the entire propilin leader peptide. Using propilin processing as a measure of propilin insertion into the plasma membrane, we found that inhibition or depletion of SecA and SecY does not affect propilin maturation. Addition of a general membrane perturbant such as ethanol also had no effect. However, dissipation of the proton motive force did cause a marked inhibition of propilin processing, indicating that membrane insertion requires this energy source. We propose that propilin insertion in the plasma membrane proceeds independently of the SecA-SecY secretion machinery but requires the proton motive force. These results present a model whereby propilin insertion leads to processing by leader peptidase B to generate the 7-kDa peptide, which is then acetylated in the presence of TraX.

F-pilus filaments extend from the surface of bacteria carrying plasmid F to initiate intercellular contacts with recipient cells and allow the conjugative transfer of the F plasmid. Several F-encoded gene products are required for the synthesis and assembly of the pilus. Mutations in these genes lead to a loss of piliation and to transfer deficiency (8).

F pili are assembled from a pool of F-pilin subunits localized in the cytoplasmic membrane of F^+ strains (16, 26–28). Subunits are derived from the 121-amino-acid precursor polypeptide, propilin, encoded by the F transfer region gene, *traA* (9).

The F-pilin maturation pathway exhibits a number of interesting features. Efficient maturation is dependent on a small membrane protein encoded by the F *traQ* gene (11, 16, 20, 25, 42, 43), although the role of TraQ is not known. N^{α}-acetylation, a relatively rare protein modification in prokaryotes, depends on the activity of another F plasmid gene, *traX*, which encodes two inner membrane proteins (3, 21, 25). Mature subunits are 70-amino-acid, N^{α}-acetylated polypeptides with both the amino and carboxy termini extending into the periplasm (Fig. 1A) (8, 34). Furthermore, a subset of F-pilin subunits appears to carry an additional modification mediated by host activities (20).

The signal peptide of propilin is also unusual. The N-terminal amino acid of mature pilin is propilin residue 52. The processing site, A–M–A-51 ↑ A-52, is typical of leader peptidase B (LepB) cleavage sites and is preceded by a 20- to 24-amino-acid sequence that has all of the features characteristic of prokaryotic secretory signal sequences (9, 38). Remarkably, however, this is distal to an equally long amino-terminal sequence that includes a series of positively charged residues. Although the N-terminal sequences of the mature pilin subunits expressed by F-like plasmids exhibit group-specific variations, the N-terminal 51-amino-acid propilin sequence has been very highly conserved (6, 7).

In this study we investigated whether LepB is responsible for the removal of the N-terminal sequences of propilin. We also examined the dependence of propilin translocation on components of the secretory pathway (required for the export of most signal-sequence-bearing proteins). In addition, we tested the requirement for the proton motive force (PMF) to drive propilin translocation. These studies show that propilin processing is dependent on LepB and does require a strong PMF. They also indicate that translocation is *secA-secY* independent despite the presence of an embedded signal sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* strains used in this study are listed in Table 1.

Plasmid pKI158 (20) constitutively expresses *traQ*. Plasmid pKI500 (22) allows cloned sequences to be expressed from a λ p_L promoter. Plasmids pKI507 and pKI503N are derivatives of the vector pKI500 and express F *tra* genes from a λ p_L promoter. Since these plasmids also express the temperature-sensitive λ *c*I857 repressor, *tra* product synthesis is repressed at 30°C and induced at 42°C (Fig. 1B). Since pKI507 and pKI503N both carry the F *tra* sequence which extends from the *Eco*RV site in *traJ* to the *Hpa*I site in *traE*, both can express the F *traY*, *traA*, and *traL* genes. Plasmid pKI503N also expresses the F *traQ* gene, which is located distal to the partial *traE* sequence on this construct (Fig. 1C).

Media and chemicals. The enriched medium was LB broth (24), and the minimal medium used for [^{35}S]methionine labeling of cells was JMM medium (22).

Sodium azide and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Company (St. Louis, Mo.).

Gene expression and in vivo labeling experiments. Genes to be expressed from a λ p_L promoter were cloned into plasmid pKI500, which carries the promoter as well as a functional copy of the λ *c*I857 repressor gene. Thus, cells were grown at 30°C in JMM medium containing antibiotics selective for maintenance of the plasmid. At an optical density at 550 $_{nm}$ of 0.4, gene expression was induced by placing culture flasks in a 42°C water bath for 10 min prior to labeling. Since prior studies have shown that propilin is rapidly degraded (20), samples (0.5 ml) were labeled with 5 μCi of [^{35}S]methionine for only 1 min and were immediately precipitated with 10% trichloroacetic acid. After centrifugation for 10 min, the

* Corresponding author. Present address: National Cancer Institute, Laboratory of Molecular Biology, Bldg. 37, Room 2D26, 37 Convent Dr., Bethesda, MD 20892-4255. Phone: (301) 496-4894. Fax: (301) 496-2212.

† Deceased.

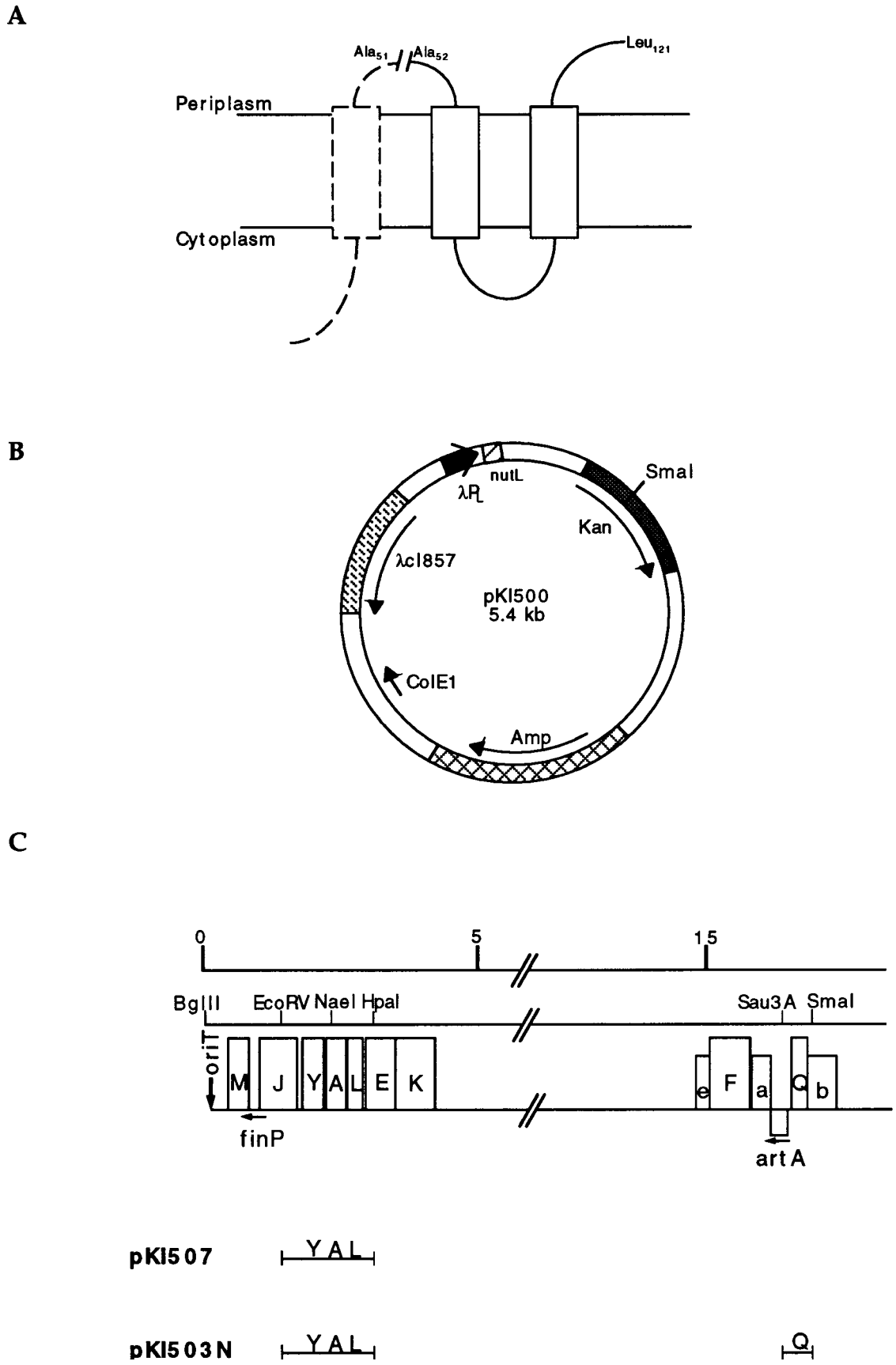

FIG. 1. Topology of F pilin, circular map of plasmid pKI500, and linear map of the F *tra* region. (A) Schematic diagram of propilin as proposed by Paiva et al. (34). Dashed lines, 51-amino-acid propilin leader peptide; -//-, potential LepB cleavage site; Ala-52, amino acid that is acetylated to yield mature F pilin. (B) Plasmid pKI500 is the vector used to express *tra* sequences from the λ p_L promoter. Cloning used the unique *Sma*I site (the map is not to scale). (C) Partial linear map of the *tra* operon. Sequences cloned in pKI500 are marked below the map. The scale is in kilobases. Restriction sites used in cloning are shown.

TABLE 1. *E. coli* strains

Strain	Relevant genotype	Reference(s)
IQ85	MC4100 *secY24 zhd-33*::Tn*10*	37
IQ86	MC4100 *zhd-33*::Tn*10*	37
IT41	W3110 *lep-9 zff*-Tet	10
IT42	W3110 *zff*-Tet	10
MM52	MC4100 *secA*	30, 31
TB1	JM83 *hsdR* (r_K^- m_K^+)	13

supernatant was removed by aspiration, and the pellet was washed with acetone, dried, and kept frozen prior to analysis.

Fractionation and detection of labeled proteins. Prior to fractionation, samples were suspended in 200 μl of sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min, and 5 μl was analyzed in each lane. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography were performed as described previously (26–28) except that a Bio-Rad Protean II apparatus was used. The gels contained an 11 to 16% exponential gradient of acrylamide formed with an 11-ml mixing volume; a constant power of 10 W was applied for 4.5 h.

RESULTS

Construction and in vivo expression of pKI507 and pKI503N. Plasmid pKI507 carries the *traYAL* sequence, whereas plasmid pKI503N carries the *traYALQ* sequence. The labeled products observed after induction of these plasmids are consistent with prior results demonstrating that propilin processing is highly dependent on TraQ (Fig. 2). The primary *traA* product detected after temperature induction of pKI507 was the 13-kDa propilin, although some amount of 7-kDa peptide is observed. The 7-kDa pilin polypeptide was the primary *traA* product detected after temperature induction of pKI503N. The *traL* product was never detected, a finding consistent with previous observations in this laboratory.

After the temperature induction of pKI507 (*traA*$^+$) and a 1-min pulse, the amount of labeled *traA* product decreased to below detectable levels 5 min after the start of the chase period, while the 7-kDa product expressed after induction of pKI503N (*traA*$^+$ *traQ*$^+$) was stable throughout the 30-min chase period (data not shown). These data are in agreement with results of a previous study in which the same genes were expressed from a T7 promoter (20).

Maturation of propilin depends on host leader peptidase B. Frost et al. (9) suggested that the context of the cleavage sequence of the propilin leader peptide appears to be typical for an *E. coli* LepB target site. Therefore, processing of the propilin leader peptide was examined under conditions in which LepB activity was impaired. Since LepB is essential for cell viability, strain IT41 ($LepB_{ts}$) is viable at 30°C but not at 42°C. After 10 min of growth at 42°C, exported peptides are no longer processed; they accumulate and impede cell growth (10). Polypeptides expressed from plasmid pKI503N were labeled for 1 min after cells had been grown at 42°C for different lengths of time. Figure 3 shows that propilin processing was unaffected in strain IT42 (wild type) and that the 7-kDa peptide was the primary *traA* product detected. However, in strain IT41 ($LepB_{ts}$), propilin processing was markedly inhibited shortly after the shift to 42°C, and the 13-kDa peptide became the predominant *traA* product. Similarly, the β-lactamase precursor used as an internal control was normally processed in IT42 but was more than 80% unprocessed in IT41, a result that confirms that LepB function was inhibited (data not shown).

No peptides of sizes intermediate between 7 and 13 kDa were observed, suggesting that the removal of the leader peptide occurs in a single step catalyzed by LepB. This is further confirmed in the accompanying paper (19).

The active site of LepB is exposed at the periplasmic side of the cytoplasmic membrane (2). Therefore, the propilin-processing assay can be used to monitor the insertion of propilin into the inner membrane and to determine whether translocation requires the components of the secretory pathway. This type of assay has been widely used by other investigators to monitor protein translocation (1, 5, 10, 15, 18, 31, 33).

Maturation of propilin is SecA independent. To examine the dependence of propilin processing on a functional *sec* system, we monitored processing under conditions that impair the *sec* machinery. Oliver et al. (32) have shown that the ATPase activity of SecA is extremely sensitive to inhibition by azide. We tested whether propilin maturation shows the high degree of sensitivity to azide expected for SecA-dependent translocation. Host strain TB1 carrying plasmid pKI503N was used to monitor the maturation of the *traA* product in the presence of TraQ at various azide concentrations. Sodium azide at 0, 1, 2, 5, and 10 mM was added prior to the 1-min labeling period, as described in Materials and Methods. Figure 4 shows that up to 10 mM azide had no adverse effects on propilin maturation: the 7-kDa peptide remained the primary product detected after induction of *traA* from plasmid pKI503N. Normally, 3 mM azide is inhibitory to cell growth because of the accumulation of nontranslocated proteins (32). β-Lactamase processing was completely inhibited under these conditions (data not shown). We further tested the involvement of SecA by monitoring the maturation of labeled propilin at the nonpermissive temperature in host strain MM52 ($SecA_{ts}$) (30). Incubation for up to 6 h at the nonpermissive temperature did not affect propilin processing, and the 7-kDa polypeptide remained the

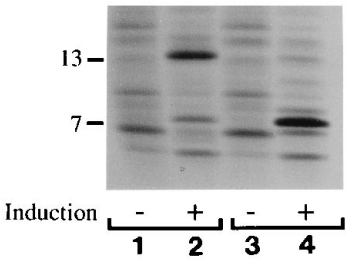

FIG. 2. Radiolabeled polypeptides expressed from plasmids carrying *tra* sequences. Lanes 1 and 2, products expressed in strain TB1 from pKI507 before (−) and after (+) induction at 42°C; lanes 3 and 4, products expressed before (−) and after (+) induction of plasmid pKI503N. The positions of the 13-kDa propilin and of the 7-kDa pilin polypeptide are marked.

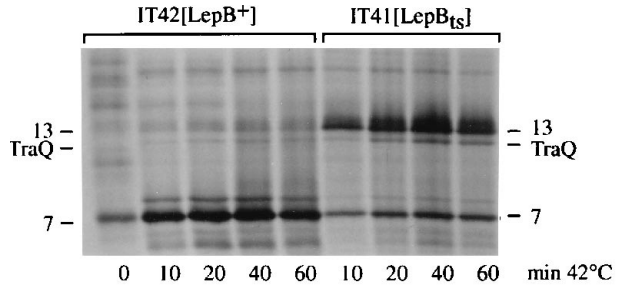

FIG. 3. Processing of propilin in wild-type and $LepB_{ts}$ strains at the nonpermissive temperature. Cultures of cells carrying plasmid pKI503N were grown at 30°C to an optical density at 550 nm of 0.4 before the shift to 42°C. Cultures were labeled for 1 min with [^{35}S]methionine and sampled immediately (0 min) or incubated for 10 to 60 min at 42°C prior to labeling. The positions of the TraQ, 13-kDa, and 7-kDa products are indicated. The 8-kDa peptide appearing after induction of pKI503N has not been investigated but may be a form of modified pilin as described previously (20).

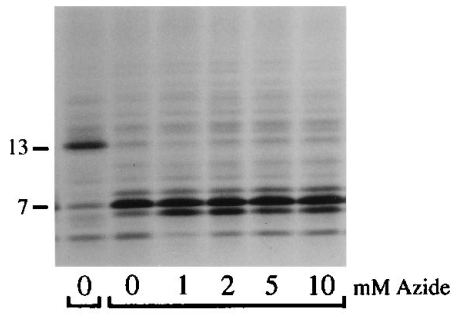

FIG. 4. Effects of SecA inhibition on propilin processing. Cells carrying plasmid pKI503N were induced at 42°C for 5 min and incubated with sodium azide (0 to 10 mM) for an additional 5 min prior to the 1-min labeling period. The propilin product expressed from *traA* is shown as a control in the leftmost lane. The positions of the 13-kDa (propilin) and 7-kDa (pilin) markers are shown on the left.

primary product detected (data not shown). Thus, propilin maturation is independent of SecA.

Propilin maturation is also SecY independent. Since propilin maturation is SecA independent, it is possible that TraQ could provide an alternative pathway for presenting propilin to the SecY-SecE-SecG translocase. Precursors of exported proteins begin to accumulate in strain IQ85 ($SecY_{ts}$) within 2 h of growth at the nonpermissive temperature of 42°C (37). Thus, to examine the dependence of propilin processing on a functional SecY protein, plasmid pKI503N was used to transform host strains IQ85 ($SecY_{ts}$) and IQ86 ($SecY^+$). Cultures were grown at 30°C to an optical density at 550 nm of 0.4 prior to the shift to 42°C. At 10, 30, 60, 90, and 120 min after the shift, gene products were labeled for 1 min and analyzed by SDS-PAGE. Figure 5 shows only the lower part of the gel. In the left panel, propilin processing was clearly not impaired, and it remained comparable to the processing of propilin seen in the *secY*$^+$ strain in the right panel of Fig. 5. More than 80% of the β-lactamase precursor was unprocessed under these conditions, indicating that secretion was effectively inhibited (data not shown). These results suggest that propilin translocation is independent of SecY. The participation of TraQ may provide an alternative translocation pathway for propilin.

Ethanol does not inhibit processing of propilin when TraQ is present. Ethanol is a membrane perturbant that can be used at concentrations greater than 8.3% to inhibit protein translocation (17, 35). We examined propilin processing in the presence of TraQ and in the presence and absence of ethanol (Fig. 6). We used the λ p_L promoter to express *traA* and *traQ* from plasmid pKI503N in TB1 host cells. After an 8-min induction at 42°C and a 2-min incubation at 37°C, ethanol was added to a 9% final concentration prior to labeling. Propilin processing was unaffected by the presence of ethanol, and the 7-kDa product remained the predominant peptide expressed from plasmid pKI503N. However, under these conditions, β-lactamase processing was blocked (Fig. 6, lanes 3 and 4), indicating that normal *sec*-dependent secretion was effectively inhibited. The data suggest that membrane translocation of propilin proceeds in an ethanol-resistant pathway.

Propilin processing requires the PMF. Protein translocation across the plasma membrane of *E. coli* requires the energy provided by the PMF (40). The presence of an ionophore, such as CCCP, was shown to inhibit the processing of the B subunit of the heat-labile enterotoxin in *E. coli* (35). We examined the processing of propilin in the presence of various concentrations of CCCP. Since TraQ is an inner membrane protein (42), its insertion may be affected by PMF depletion. Therefore, to rule out indirect effects of CCCP on propilin processing via effects on TraQ localization, we used plasmid pKI158 to express *traQ* constitutively in MC4100. These cells also carried plasmid pKI507 (*traA*). They were induced at 42°C for 5 min, and CCCP was added to concentrations of 25 and 50 μM (Fig. 7). Most of the newly synthesized *traA* products were in the 13-kDa precursor form. These results indicate that propilin translocation, as measured by processing inhibition, is highly dependent on the PMF of the host cell.

DISCUSSION

The propilin signal sequence resembles a typical *sec*-dependent, LepB-cleavable signal sequence but is embedded within a larger leader peptide. Thus, it is not exposed at the N terminus of the propilin molecule. This unusual configuration of the leader peptide is conserved among various IncF groups (8) and may also be found in the *Agrobacterium* conjugative *virB* system (12). The requirement for TraQ for pilin maturation raised the possibility that removal of the TraA leader is catalyzed not by LepB but, instead, by TraQ. However, our results show that expression of the *traA* gene in a $TraQ^+$ $LepB_{ts}$ background, at the nonpermissive temperature, causes inhibition of propilin processing. Since we did not observe significant amounts of peptides with molecular masses between 13 and 7 kDa, this suggested that TraQ did not cleave within the leader peptide region of propilin to expose the embedded *sec*-distinctive signal sequence. The presence of small amounts of 7-kDa peptide, in a LepB mutant, at the nonpermissive temperatures possibly is due to an incomplete inactivation of LepB or to cleavage by another host protease. In the accompanying paper we show that removal of the 51-amino-acid leader peptide occurs by a single polypeptide cleavage event (19).

We examined the roles of various host factors in the translocation of propilin. We also examined the role of the F protein, TraQ, and its possible delivery of propilin to the secretory system. Our results argue against the involvement of any component of the *sec* system in the targeting and processing of propilin. First, propilin translocation showed no sensitivity to azide as would be expected with SecA-dependent translocation. Second, temperature-sensitive mutations in SecA and SecY did not affect propilin processing at the nonpermissive temperature. Third, TraQ has no significant homology to *sec*-encoded proteins that interact with SecY. Fourth, in vivo translocation of propilin is not inhibited by a membrane perturbant such as ethanol. Processing of β-lactamase (Fig. 6, lanes 3, 4) and of various periplasmic F *tra* products (synthesized with typical amino-terminal signal sequences) is inhibited by etha-

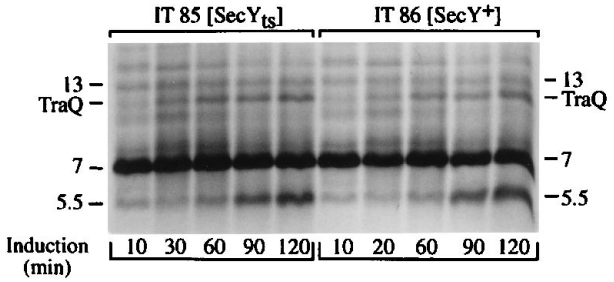

FIG. 5. Analysis of the *traA* products expressed in $SecY_{ts}$ cells at the nonpermissive temperature. The autoradiograph shows products expressed from plasmid pKI503N in wild-type cells (IT86) and $SecY_{ts}$ cells (IT85). Cells were induced at 42°C for 10 to 120 min prior to the 1-min labeling pulse. The positions of TraQ and of the TraA-derived 13-, 7-, and 5.5-kDa polypeptides are indicated.

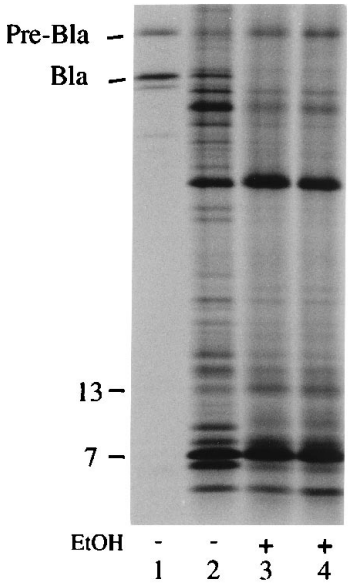

FIG. 6. TraA products synthesized in the presence of 9% ethanol. Cells carrying plasmid pKI503N were induced at 42°C for 8 min and then equilibrated at 37°C for 2 min. The cells were incubated for a further 2 min without ethanol (EtOH) (lane 2) or with 9% ethanol for 2 min (lane 3) or 4 min (lane 4) and then labeled. The positions of the 13- and 7-kDa molecular mass markers are shown. Pre-Bla and Bla, β-lactamase precursor and mature products, respectively, present in the first lane. The large induced band is a fusion protein resulting from the insertion of *tra* sequences into the *kan* gene on pKI500.

nol (8, 22, 23, 42, 43). Thus, we conclude that TraQ either alters the functions of SecA and SecY by suppressing the temperature sensitivity specifically for propilin maturation or, more likely, provides an alternate route for propilin translocation that is completely independent of the SecA-SecY secretion pathway.

Pilus synthesis is essential for F conjugation (8). Thus, correct subunit translocation and pilus extension are critical parts of the process. Depletion of the PMF causes inhibition of propilin processing, indicating that the inability to assemble a pilus will block F conjugation. The effects of PMF depletion and the requirements for the SecA-SecY secretion pathway for the export of other F *tra* proteins remain to be investigated.

The PMF is required for *sec*-dependent and -independent protein translocation, and ionophores such as CCCP have severe inhibitory effects on secretion (35, 39). On the other hand, the mechanism of ethanol inhibition remains unclear. Palva et al. (35) suggested that ethanol acts like dinitrophenol and CCCP and dissipates the PMF. However, our results indicate that these compounds have distinctly different effects on propilin and β-lactamase processing, suggesting that ethanol and CCCP act differently or affect different stages of the translocation pathway. Moreover, previous studies used periplasmic proteins, which have to cross the plasma membrane barrier (1, 4, 15, 18, 33, 36), whereas propilin and other integral membrane proteins do not. Therefore, proteins may be affected differently by membrane perturbants, with CCCP possibly affecting membrane insertion and translocation and ethanol affecting translocation only.

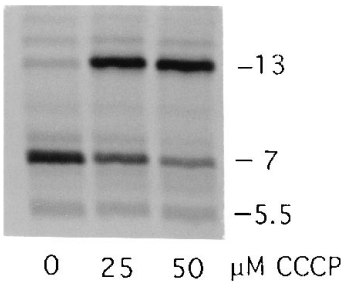

FIG. 7. Effects of CCCP-induced depletion of the PMF on the processing of propilin. Cells carrying plasmids pKI158 (*traQ* constitutive expression) and pKI507 (*traA*) were induced at 42°C for 5 min. The cells were incubated for a further 5 min without CCCP or with 25 or 50 μM CCCP. Products were labeled for 1 min. The positions of the 13- and 7-kDa molecular mass markers are shown.

Membrane insertion of certain hydrophobic domains can be a *sec*-independent event (29, 39). However, *sec*-independent membrane insertion of signal-sequence-bearing proteins is rare. The procoat protein of phage M13 (gp VIII) is one such example. The procoat protein has a typical signal sequence that is cleaved by LepB. However, the single transmembrane domain of the mature polypeptide inserts spontaneously in the cytoplasmic membrane, and this insertion does not require any additional phage-encoded activities (14, 41). Thus, TraQ-mediated translocation of propilin may represent a novel export pathway in which TraQ acts either by forming an inner membrane protein channel or by serving as a chaperone that impedes premature propilin folding or aggregation. In either case, the lack of TraQ seems to prevent efficient insertion into the cytoplasmic membrane. Because of the unusual configuration of the propilin leader peptide and because of the requirement for TraQ, it is conceivable that the propilin-processing requirement for TraQ is inherent to that leader peptide. This possibility is investigated in the accompanying paper (19).

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